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Short Communication

Enantioselective high-performance liquid chromatographic method for the determination of methadone in serum using an AGP and a CN column as chiral and analytical column, respectively

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ABSTRACT

A simple and sensitive HPLC method with ultraviolet absorption detection at 200 nm is described for the determination of methadone enantiomers in human serum, using dextropropoxyphene as an internal standard and organic solvent extraction. Separation was performed on two serially coupled columns, CN and Chiral AGP, with a mobile phase consisting of acetonitrile, dimethyloctylamine and phosphate buffer. Using 1.0 ml of serum, 5 nmol/l of each enantiomer could be determined with an acceptable precision. No interactions from several drugs were observed. The method has been successfully used in a pharmacokinetic study. More than 2500 serum samples have been separated on the same AGP column with acceptable selectivity and resolution.

INTRODUCTION

Methadone is a synthetic analgesic agent widely used in the treatment of narcotic addiction and severe pain. Methadone as used clinically in Denmark is a racemic mixture of two enantiomers, (*R*)-(−)- and (*S*)-(+)-methadone. (*R*)-(−)-Methadone has been estimated to be 50 times more potent than (*S*)-(+)-methadone in a human analgesic study [1].

Individual doses are necessary for optimum clinical results because of inter-individual differences in the pharmacokinetics of total methadone. With the purpose of examining the possible stereoselective absorption and disposition of methadone, we need a simple and sensitive method for the measurement of the enantiomers in serum.

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Enantioselective determination of methadone can be achieved by GC after derivatization [2], but a direct HPLC method based on Chiral-AGP seems more suitable for large pharmacokinetic studies [3,4]. Previously described methods have either an insufficient sensitivity [3] or are too expensive, because the Chiral-AGP column has a short lifetime [4].

This paper describes a simple and economical method based on stereoselective HPLC separation of methadone enantiomers in serum by using a Sperisorb CN analytical column coupled in series with a Chiral-AGP column and UV detection at 200 nm. The sensitivity, precision and accuracy are satisfactory for pharmacokinetic studies in patients treated even with low doses of methadone.

EXPERIMENTAL

Chemicals

rac-Methadone hydrochloride (pharmacopoeial grade) was supplied by the hospital pharmacy, Bispebjerg Hospital. (*R*)-(−)-methadone hydrochloride (Levomethadone hydrochloride) and dextropropoxyphene hydrochloride were gifts from Hoecht (Frankfurt/Main, Germany) and Eli Lilly (Indianapolis, IN, USA), respectively. Stock standard solutions were prepared in ethanol–water (50:50, v/v). Acetonitrile was of LiChrosolv grade and *n*-hexane was of Uvasol grade from Merck (Darmstadt, Germany). Dimethyloctylamine (DMOA) was obtained from K & K Labs. (Cleveland, OH, USA). All other chemicals were of analytical-reagent grade.

Venous blood samples were drawn into plain Vacutainer tubes from Becton/Dickinson (Meylan, France).

Apparatus

The HPLC system consisted of a Waters (Milford, MA, USA) automatic system with WISP 710 sample processor, Model 6000 A pumps and Model 720 system controller, a variable-wavelength UV–Vis spectrophotometer detector, a.u.f.s. 0.0005, from Chrompack (Middelburg, Netherlands) and a D-2000 Chromato-Integrator from Merck–Hitachi (Darmstadt, Germany).

The analytical column, ChromSep Sperisorb CN, 5 μm (100 × 3 mm I.D.), and the reversed-phase guard column from Chrompack were coupled in series with a Chiral-AGP chiral column (100 × 4 mm I.D.) from ChromTech (Norsborg, Sweden).

Chromatographic conditions

The detector was set at 200 nm. The mobile phase was 10 m*M* sodium phosphate buffer (pH 5.0)–acetonitrile–DMOA (900:100:0.5) at a flow-rate of 0.9 ml/min. The percentage of acetonitrile was lowered as the column became older (see Results and Discussion). The mobile phase was degassed with helium for 15 min before and during use.

Procedures

A 1.0-ml aliquot of 1 *M* sodium carbonate buffer (pH 10.0) containing 160 n*M* dextropropoxyphene was added to 1.0 ml of serum. The sample was extracted with 6.0 ml of *n*-hexane by horizontal shaking for 15 min. After centrifugation for 5 min at 1300 *g* and cooling in a dry-ice–acetone bath for 10 min, the organic phase was decanted into brown glass tubes and evaporated to dryness at room temperature under nitrogen. The residue was dissolved in 100 μl of mobile phase and 10–80 μl were injected into the HPLC system.

Calibration samples were prepared by spiking blank human blood serum with known amounts (0–1200 nmol/l) of racemic methadone. Quantification was achieved by comparing the peak-height ratios of unknown samples with those obtained for the calibration samples. Three quality control samples were analysed on each day of analysis.

RESULTS AND DISCUSSION

The method described offers an alternative to the already existing chiral HPLC methods [3,4] for separating methadone enantiomers. We found that addition of an organic modifier such as DMOA was necessary to obtain a satisfactory peak shape. With this modifier, the Chiral-AGP column had to be supplemented with a cyano-bonded analytical column in order to obtain

satisfactory selectivity from co-extracted compounds. The limit of quantification in human serum is 5 nmol/l (1.7 ng/ml) for each enantiomer (relative standard deviation less than 20%). This is better than that reported by Beck *et al.* [3] and slightly lower than that reported by Schmidt *et al.* [4], because the use of 1.0 ml of serum rather than 2.0 ml of plasma [4] may be an advantage in pharmacokinetic studies. Better sensitivity is obtained by UV detection at 200 nm, which is possible when acetonitrile and a low concentration of organic modifier are used in the mobile phase.

The chromatograms obtained from human serum samples showed a good separation of (*R*)-(−)- and (*S*)-(+)-methadone and the internal standard from co-extracted compounds, the resolution factor between (*R*)-(−)- and (*S*)-(+)-methadone being not less than 1.7 (Fig. 1). On spiking serum with pure (*R*)-(−)-methadone, no racemization was seen during the analytical procedures. The recoveries after extraction with *n*-hexane were about 90% for (*R*)-(−)- and (*S*)-(+)-methadone and dextropropoxyphene.

The assay linearity for the enantiomers was determined by performing linear regression analysis on the plot of the peak-height ratios of either (*R*)-(−)-methadone or (*S*)-(+)-methadone to the internal standard (*y*) *versus* concentration (*x*) in the range 10–600 nmol/l (concentration range for each enantiomer), the calibration graphs typically being $y = 0.0091x - 0.0607$ ($R^2 = 0.9987$) and $y = 0.0083x - 0.0476$ ($R^2 = 0.9996$). Accuracy was monitored in seventeen patients receiving racemic methadone. Blood samples were collected at various times after dosage. The determination of (*R*)-(−)- and (*S*)-(+)-methadone concentration by this method was summarized and compared with the result of a total methadone determination by a conventional GC method [5] (Fig. 2), the equation being [GC] = [HPLC] · 1.03 + 6.7 nmol/l, with a correlation coefficient of 0.9654.

The selectivity was monitored by analysing serum from patients undergoing treatment with drugs generally used in the treatment of severe pains *viz.*, benzodiazepines, morphine, ketobemidone, carbamazepine, valproic acid, piroxicam and tenoxicam. No interfering peaks were seen.

The reproducibility was determined by analys-

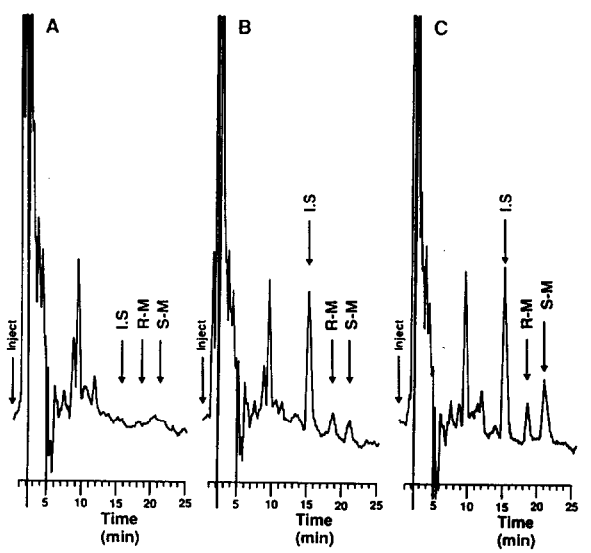

Fig. 1. Chromatograms of human serum extracts analysed as described. (A) Patient blank serum (40 μl injected.); (B) human serum spiked with 50 nmol/l of racemic methadone [30 μl injected. At lower concentrations 80 μl were injected (see *Procedures*)]; (C) patient serum 2 h after a dose of 40 mg of racemic methadone administered orally (30 μl injected). I.S. = internal standard (dextropropoxyphene); R-M = (*R*)-(−)-methadone; S-M–(*S*)-(+)-methadone.

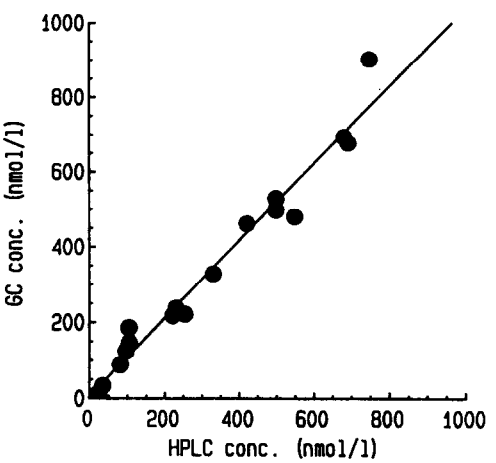

Fig. 2. Serum samples from seventeen patients receiving racemic methadone were analysed by the proposed method and by a conventional GC method [5].

TABLE I

REPRODUCIBILITY OF REPLICATE ANALYSES OF METHADONE ENANTIOMERS ADDED TO HUMAN SERUM

Added		Measured			
Compound	Concentration (nmol/l)	Serum concentration of (*R*)-(−)-methadone[a] (nmol/l)	R.S.D. (%)	Serum concentration of (S)-(+)-methadone[a] (nmol/l)	R.S.D. (%)
rac-Methadone	51	26	11.9	25	11.7
rac-Methadone	205	99	11.3	97	6.7
rac-Methadone	359	182[b]	4.6	175[b]	8.2
rac-Methadone	512	258[b]	6.6	251[b]	5.2
rac-Methadone	767	360[c]	6.4	352[c]	7.6
(*R*)-(−)-methadone	106	111	5.0		

[a] Mean values from eight duplicate samples at each concentration, except where indicated otherwise.
[b] $n = 6$.
[c] $n = 9$.

ing spiked serum samples at random on different days (Table I). The results of the method were satisfactory, and demonstrate the stability of methadone in samples stored at −20°C from 1 day to 3 months.

The retention times were 15.2, 18.5 and 21.1 min for dextropropoxyphene and for (*R*)-(−)- and (*S*)-(+)-methadone, respectively. After some time the capacity of the AGP column decreased but could be regenerated by recirculating 10 n*M* phosphate buffer (pH 7) for at least 48 h. Alternatively, a mobile phase with a lower percentage of acetonitrile (5–8%) will restore the retention times with an acceptable selectivity and resolution.

By using an organic modifier and the CN column as the analytical column before the AGP column, the latter column can be used to separate more than 2500 serum samples, provided that the above-mentioned regeneration procedure and mobile phase modifications are carried out.

The method has been successfully used in pharmacokinetic studies of methadone enantiomers. A dose of 20 mg of racemic methadone was administered intravenously to a patient suffering from severe angina pectoris and 48 h later an oral dose of 40 mg of racemic methadone was administered. The serum concentration *versus* time profile of (*R*)-(−)- and (*S*)-(+)-methadone is shown in Fig. 3.

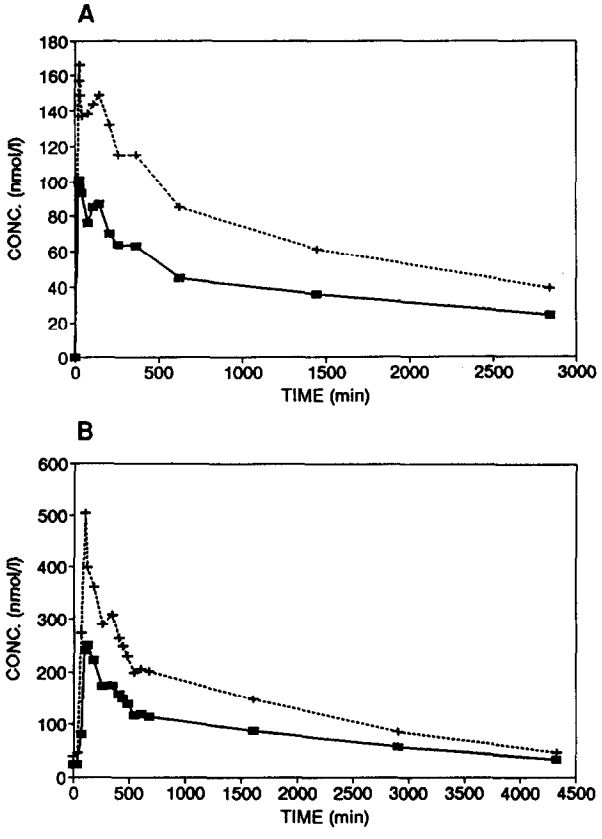

Fig. 3. Serum concentration profile of (*R*)-(−)- and (*S*)-(+)-methadone in a female patient receiving (A) and initial dose of 20 mg of racemic methadone intravenously at time zero and (B) 48 h later a dose of 40 mg racemic methadone orally. ■ = (*R*)-(−)-methadone; + = (*S*)-(+)-methadone.

In conclusion, the proposed method provides a good alternative to the two recently published chiral HPLC assays. The extended lifetime of the AGP column together with an acceptable limit of quantification and good accuracy and precision make this method suitable for extensive pharmacokinetic studies.

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